STARCHES OF ENDOSPERMS POSSESSING DIFFERENT ALLELES AT THE *AMYLOSE-EXTENDER* LOCUS IN *ZEA MAYS* L.[1]

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ABSTRACT

Starches from seven independently occurring *amylose-extender* (*ae*) maize (*Zea mays* L.) alleles, named *B2, B3, il, i2, M1, M2,* and *Ref,* were compared. Apparent amylose content of *il* and *i2* starch granules averaged 56.6% and differed from that of the other five alleles which averaged 64.5%. Starch granules were subjected to aqueous leaching in boiling water, and the 40% of the total starch solubilized was separated into butanol complexing and noncomplexing fractions. Amylose was preferentially leached from the granules but not all was solubilized. The butanol complexing fraction from all *ae* alleles had similar physicochemical properties which were typical of amylose. Properties of the butanol noncomplexing fraction were consistent with the theory that this material was a mixture of short-chain amylose and typical amylopectin. The *il* and *i2* noncomplexing fractions had a lower percentage conversion to maltose by β-amylase, a lower apparent amylose content, a lower polysaccharide-iodine absorbance maximum, and a higher ratio of total to reducing glucose units than the other five *ae* alleles. The *il* and *i2* starches appeared to have the same components as the other *ae* alleles but with lesser amounts of short-chain amylose. Polysaccharide composition of the starch for all *ae* alleles examined was similar to that produced by the allele currently used in amylomaize production. Starches of the *B2, B3, M1, M2,* and *Ref* alleles would be suitable for current industrial utilization, and the *il* and *i2* alleles might also be useful.

Maize (*Zea mays* L.) types containing starch with increased amylose content became desirable in the late 1940s with the realization of the value of films, fibers, and other industrial products that could be made from amylose (1). High-amylose maize became feasible with the discovery of the *amylose-extender* (*ae*) gene in the early 1950s (2,3). Starch with amylose content twice that found in *normal* maize was produced by this gene. Since this observation, maize lines homozygous for the *ae* gene have been extensively evaluated and selected to develop high-amylose corn hybrids (amylomaize) having as high as 80% amylose; however, as types with higher amylose contents were developed, processors have desired higher amylose contents than were currently available (4). Furthermore, the starch processing industry has expressed an interest in discovering other starch types with specialized properties.

Possible sources of different starch types which have not been evaluated include the different mutations that have independently occurred at the *ae* gene locus. Since five independently arising *ae* alleles have been shown to be located at different points within the *ae* gene (5), a potential exists for kernels homozygous for any one of these alleles to have a starch with unique properties.

All researchers agree that *ae* starch has an increased amylose percentage;

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however, no agreement exists on the precise structure of the amylose and amylopectin components. Fractionation of amylomaize starch into amylose, that portion of the starch precipitated by a low-molecular-weight alcohol, and amylopectin, that portion not precipitated, resulted in the observation by numerous researchers (reviewed by 6,7) that the physicochemical properties of the amylose component were similar to those obtained from *normal* maize starch, while those of the amylopectin fraction were not. Wolff *et al.* (8) concluded that the structure of the branched or amylopectin component was representative of a new polysaccharide type, intermediate in structure between amylose and amylopectin with inner and outer chain lengths of 13 and 23 glucose units, respectively, compared to 10 and 17 for *normal* maize amylopectin. They stated that this fraction was not a mixture of high- and low-molecular-weight components. Mercier (9), however, concluded that the polysaccharide found in the amylomaize amylopectin fraction is branched but that the material has its own structure with longer inner chains (Degree of Polymerization (DP) above 60) compared to those of *waxy* maize amylopectin (DP = 30). A third explanation for the unusual properties of the *ae* amylopectin fraction, offered by Greenwood and coworkers (6,7,10,11), is that this fraction contains both normal amylopectin and short-chain amylose molecules that are too small to be precipitated by alcohol complexing agents.

Banks, Greenwood, and Muir (7,12) suggested that *ae* amylopectin could best be studied by using the resistance of *ae* starch granules to swelling in hot aqueous solution. To demonstrate, they leached two amylomaize starches for 1 hr in boiling water, removed the residual unsolubilized starch granules, saturated the supernatant solution containing the leached polysaccharide with butanol, and recovered the resulting complex. The butanol complexing polysaccharide was considered to be typical amylose, and the butanol noncomplexing fraction was similar to the previously purified short-chain amylose from amylomaize (11).

This study was conducted to determine the similarity of starches from endosperms homozygous for each of seven *ae* alleles by using the aqueous leaching procedure (12).

MATERIALS AND METHODS

Genetic Material

Seven *ae* alleles, termed *ae-B2* (Bear-two), *ae-B3* (Bear-three), *ae-i1* (induced-one), *ae-i2* (induced-two), *ae-M1* (Missouri-one), *ae-M2* (Missouri-two), and *ae-Ref* (Reference), were used (5). The seven alleles will be denoted by their allele names, *B2*, *B3*, *i1*, *i2*, *M1*, *M2*, and *Ref*, while the symbol *ae* will be used to refer to the locus in general. The *ae* allele found by Kramer *et al.* (2) is termed *Reference* in accordance with maize nomenclature recommendations (13) because it was the first allele made generally available to researchers. These *ae* alleles were backcrossed six times to the W64A inbred, with the exception of *M2*, which was backcrossed four times. The *normal* (*AeWx*) W64A inbred and the *waxy* (*wx*) mutant, backcrossed six times to W64A, were included when appropriate as controls. These genotypes were also studied as backcross-six conversions to the W23 inbred.

In the summer of 1970, seven *ae* mutants backcrossed to W64A and the *normal* W64A inbred were grown at Rock Springs, Pa., using a randomized complete

block design. The plants were self-pollinated; ears were harvested at maturity, dried at 37° C, and shelled; and the kernels were stored at room temperature. Four ears were analyzed for each genotype. Besides these ears, several ears grown in adjacent rows also served as a source of starch granules for which amylose content was determined. The W23 genotypes were grown in either 1970 or 1971. One ear was used for each W23 genotype, and only the percentage amylose of total starch was determined.

Starch Granule Isolation and Purification

Starch granules were isolated from approximately 5 g of kernels selected at random from each ear. The procedure of Adkins and Greenwood (14) was adapted for kernel steeping and grinding. Approximately 50 ml of steep solution (0.02*M* acetate, 0.01*M* $HgCl_2$, pH adjusted to 6.5 with NaOH) was added to the kernels. After 12 hr in a 40° C water bath, the pericarp and germ were removed and discarded. The endosperms were steeped for an additional 48 hr and then ground using a large glass mortar and pestle. After 1 min of grinding, steep solution was added, and the starch slurry was poured onto a nylon mesh screen with openings of 75 by 75 μ. Material not passing the screen was again ground, and filtering and grinding were repeated until essentially all starch granules were released. The material passing through nylon mesh with 75 by 75 μ openings was subsequently filtered through nylon mesh with openings of 44 by 44 μ. The retained particles were placed in a TenBroeck hand homogenizer and further ground. This preparation was filtered through 44 μ nylon mesh with retained material discarded.

Following isolation, the starch granules were centrifuged (2000 × *g*, 15 min), and the supernatant discarded. The starch granules were suspended in distilled water and centrifuged as before, and the supernatant was discarded. The starch granules were washed with water an additional four times as above. The washed starch granules were transferred to a 250-ml glass separatory funnel with 160 ml of 0.1*M* NaCl. Fifty milliliters of toluene was added, and the mixture was vigorously shaken for 15 min using a gyrotory water bath shaker (New Brunswick Scientific, New Brunswick, N.J.). After standing overnight at 4° C, the toluene layer was removed and layered on 675 ml of water. Three hours later the toluene layer, containing lipids and denatured protein, and most of the water was discarded. Sedimented granules were recombined with the main starch sample. Fresh toluene (50 ml) was added to the separatory funnel, and this entire procedure was repeated nine times. The starch granules were centrifuged (2000 × *g*, 15 min), the supernatant discarded, and distilled water was added. This was done four times to remove all NaCl. The purified starch granule preparation was stored refrigerated under toluene in distilled water.

Amylose Determination

The amylose determination procedure of Wolf *et al.* (15) was modified as follows. Hydrated starch granules (approximately 0.2 g dry weight) were added to 20 ml 90% dimethyl sulfoxide (DMSO) in a polypropylene centrifuge bottle which was shaken on a gyrotory shaker for 24 hr at room temperature. Following centrifugation (2000 × *g*, 10 min), 5 ml of dispersed starch was added to 15 ml of absolute ethanol in a polypropylene centrifuge bottle. The mixture was shaken for 2 hr at room temperature. The starch precipitate was collected by

centrifugation (2000 × *g*, 10 min), the supernatant discarded, 25 ml 90% DMSO added, and the bottle shaken briefly to disperse the starch. The starch-iodine solution was prepared by the potassium iodide-potassium iodate ($KI-KIO_3$) method of Wolf *et al.* (15) except that 0.0051 *M* KIO_3 was used. With this sixfold increase in KIO_3, I^- becomes limiting in formation of the starch-I_2 complex (16). To compensate for the slight instability of the starch-I_2 complex that results, the absorbance was determined 5 min after HCl addition. Absorbancy was measured at 615 nm using a Beckman Model DU-2 spectrophotometer. The extinction coefficient was calculated, and the amylose percentage estimated using the relation of Wolf *et al.* (15). Parallel tests of our procedure with that of Wolf *et al.* (15) involving various types of starch fractions showed that the procedures produced comparable results. Based on similar results for amylopectin and amylose standards, the calibration curve developed by Wolf *et al.* (15) was considered appropriate for use. For comparison, amylose content was estimated for the commercial *ae* starch preparations Amylon V and VII obtained from National Starch and Chemical Co., Plainfield, N.J., which were labeled 57 and 66% amylose, respectively. Amylose content was determined directly on starch fractions obtained from aqueous leaching.

Aqueous Leaching of Isolated Starch Granules

Starch granules isolated from seven *ae* alleles converted to the W64A inbred were leached in boiling water. Aqueous leaching was conducted using a procedure based on that of Banks *et al.* (12). Hydrated starch granules (approximately 0.5 g dry weight) were added to 100 ml distilled water in a 1-liter distilling flask. Nitrogen was passed through the flask for 5 min to displace air. The N_2 was exhausted through a condenser and water in a flask. With N_2 flow continued, the starch suspension was boiled for 1 hr just vigorously enough to keep the starch granules suspended. Following leaching, the sample was transferred to a centrifuge bottle with a 10-ml water rinse and rapidly cooled in an ice bath to 30° C. Residual starch granules, containing most of the original amylopectin, were recovered by centrifugation (2000 × *g*, 10 min, 30° C). Ten milliliters of 1-butanol was added to the supernatant. The sample then stood at room temperature for 5 hr. Following centrifugation (2000 × *g*, 10 min), the butanol complex was dispersed by adding 1 ml of boiling distilled water and 9 ml DMSO. The supernatant containing the noncomplexing polysaccharide was concentrated to 25 ml on a rotary evaporator and was precipitated by adding 4 volumes of acetone. This precipitate was collected by centrifugation as before and dissolved in 10 ml DMSO. Starch not solubilized by aqueous leaching from three of the four ears was dispersed with three 90% DMSO extractions at 70°, 25°, and 70° C, respectively.

Total Carbohydrate Determination

Carbohydrate quantitation was accomplished by enzymatic hydrolysis of the polysaccharides to glucose using glucoamylase from *Aspergillus niger* as described by Pazur (17). The glucose liberated after 20 hr hydrolysis of triplicate 1-ml samples was measured by the Nelson-Somogyi reducing sugar test (18). Absorbance was determined at 540 nm with a Beckman Model DU-2 spectrophotometer.

β-Amylolysis Procedure

β-Amylase (Nutritional Biochemicals Corporation) was used as described by Whelan (19) except that serum albumin was eliminated. Duplicate samples were layered with several drops of toluene to inhibit bacterial growth and were incubated at 35° C with occasional mixing. The liberated maltose was measured by the Nelson-Somogyi reducing sugar test (18).

Absorbance Spectra

The absorbance spectra of starch-I_2 complexes were determined using a modification of the procedure of Krisman (20). In this study, 1 ml of stock iodine-iodide solution containing 0.102*M* I_2 and 1.566*M* KI was added to 179 ml of distilled water instead of 260 ml of saturated $CaCl_2$. This I_2-KI dilution was chosen because the I_2 concentration is equivalent to that formed during the previously described amylose estimation procedure. Six parts of this solution was added to one part of carbohydrate solution and the absorption spectrum obtained from 775 to 450 nm using a Model 15 Carey spectrophotometer. All carbohydrate samples were diluted to obtain a final concentration of approximately 1.74 mg per 100 ml. The absorbance maximum and extinction coefficient at 615 nm were determined.

Ratio of Total Glucose to Reducing Glucose Units

The reducing glucose units in starch fractions obtained by aqueous leaching were measured by performing the Nelson-Somogyi reducing sugar test (18) on unhydrolyzed starch fractions. A 1-ml aliquot of the starch fraction was added to 1.5 ml water. Glucose equivalents were then determined on duplicate samples of this dilution. Using total carbohydrate content determined by the glucoamylase method, the ratio of total to reducing glucose units was computed.

Statistical Analyses

Analysis of variance was calculated using standard techniques. The properties of the butanol complexing and butanol noncomplexing fractions were compared independently to determine if differences existed between the *ae* alleles. These data were treated as a randomized complete block design with four replications. The data for amylose content of unfractionated starch were treated as a completely randomized design to permit inclusion of all ears sampled. Following analysis of variance, mean separations were performed using the procedure of Waller and Duncan (21) with $k = 100$.

RESULTS AND DISCUSSION

Amylose Content of Unfractionated Starches

The mean amylose contents determined for unfractionated starches of *ae, normal,* and *wx* maize are shown in Table I. Amylose content of starch from individual ears of the same W64A genotype differed by 2–7% which is less than that observed by Bear *et al.* (22). This reduced variation between ears of the same genotype may be owing to sampling ears that were pollinated within a 3-day period. Significant differences in amylose content were observed among *ae* alleles converted to the W64A inbred (Table I). Although significant, the differences among *B2, B3, M1, M2,* and *Ref* are quite small and of minor

importance. However, the *il* and *i2* alleles contain much less amylose than the starch of the other five *ae* alleles. This effect appears associated with the alleles and not with differences in genetic background, because all alleles except *M2* were backcrossed six times to the W64A inbred and because similar differences were observed between these *ae* alleles converted to the W23 inbred. Since only a single ear was examined for each W23 genotype, statistical significance could not be determined. However, the *il* and *i2* amylose percentages were the lowest of the *ae* alleles examined.

Normal starch amylose percentage was 1–2% higher than that observed by Wolf *et al.* (15). This may be due to inherent differences between the inbreds examined and/or to effects of cooler average temperature during the Pennsylvania growing season. As typical for the genotype, *wx* starch was essentially free of amylose. Amylose percentages for Amylon V and VII were 61.3 and 68.7%, respectively, which were 4 and 3% higher than labeled.

Amylose content determined by this procedure is only an estimate of the true amount and is termed "apparent amylose" (15). For example, if amylomaize has an unusual branched component with long side chains capable of binding iodine, the amylomaize percentage would be overestimated. Conversely, if the unusual properties of the amylomaize branched components are the result of contaminating short-chain amylose, amylose content would be underestimated.

Aqueous Leaching of Isolated Starch Granules

An average of 37.9% of the total starch from the seven *ae* alleles was solubilized by leaching for 1 hr in boiling water. The percentage of starch leached did not differ significantly between the *ae* alleles. The percentages observed in this study are in agreement with the 33, 42, and 45% starch leached from *ae* starches of different amylose content by Banks *et al.* (7,12).

The starch solubilized by aqueous leaching was fractionated into butanol complexing and noncomplexing fractions, and the amount of starch in each fraction was determined. Total carbohydrate in each of the two fractions was

TABLE I
Amylose Percentage in DMSO-Dispersed Starch Granules

Maize Genotype	Amylose Content (%)	
	W64A Inbred[a]	W23 Inbred
wx	0.1h	...
Normal	29.1g	29.9
ae allele		
B2	62.4de	73.0
B3	66.1ab	...
il	55.9f	62.3
i2	57.3f	64.3
M1	65.6b	67.6
M2	63.4cd	65.8
Ref	65.1bc	69.2

[a]Values for the W64A inbred followed by the same letter are not significantly different from each other using Duncan's Modified (Bayesian) Lease Significant Difference Test with $k = 100$.

expressed as a percentage of starch leached from the granules (Tables II and III). Although not differing significantly from every other allele, *i1* and *i2* appear to have a higher percentage of leached starch that complexes with butanol (Table II). Apparently, the same quantity of starch is accessible to solubilization from the starch granules of these alleles but, at least for *i1*, more of this leached material is considered amylose based on its ability to complex with butanol. In two studies (7,12), the butanol noncomplexing material was reported to comprise approximately 25 and 33% of the polysaccharide removed by leaching compared to the average of 49% (Table III) we observed. This discrepancy may be the result of more amylose being extracted by the aqueous leaching procedure of Banks *et al.* (7,12).

TABLE II
Summary of Physicochemical Characterizations on the Butanol Complexing Fraction Obtained by Aqueous Leaching[a]

Amylose-Extender Allele	% of Leached Starch	Conversion to Maltose by β-Amylase %	Chain Length[b]	Amylose Content (%) (KI-KIO_3 Method)	Amylose Content (%) (from I_2-KI Spectrum)	Absorbance Maximum nm
B2	46.8c	82.5a	522a	94.4a	95.8a	652a
B3	49.4bc	84.0a	467a	92.8a	94.9a	650a
i1	57.0a	81.5a	545a	92.8a	96.2a	650a
i2	53.0ab	82.1a	509a	94.2a	95.5a	650a
M1	50.8bc	84.8a	516a	95.4a	97.7a	650a
M2	50.9bc	82.2a	474a	93.2a	96.2a	651a
Ref	51.4abc	83.0a	526a	93.0a	95.3a	649a

[a]Values followed by the same letter within a column are not significantly different from each other using Duncan's Modified (Bayesian) Least Significant Difference Test with $k = 100$.
[b]Estimated as the ratio of reducing to nonreducing glucose units.

TABLE III
Summary of Physicochemical Characterizations on the Butanol Noncomplexing Fraction Obtained by Aqueous Leaching[a]

Amylose-Extender Allele	% of Leached Starch	Conversion to Maltose by β-Amylase %	Chain Length[b]	Amylose Content (%) (KI-KIO_3 Method)	Amylose Content (%) (from I_2-KI Spectrum)	Absorbance Maximum nm
B2	53.2a	70.8ab	140bc	60.3a	63.7a	591a
B3	50.6ab	72.5a	126c	59.2a	64.0a	589ab
i1	43.0c	66.3c	182a	49.3b	53.2c	586ab
i2	47.0bc	69.0b	148b	52.0b	57.0b	584b
M1	49.2ab	71.8a	126c	58.6a	62.8a	590a
M2	49.1ab	71.7a	129c	58.5a	64.0a	590a
Ref	48.6abc	72.3a	123c	58.1a	63.8a	590a

[a,b]Same as Table II.

Physicochemical characterizations of the butanol complexing fractions are given in Table II. No differences are apparent between any of the *ae* alleles, although different percentages of complexible material were extracted by leaching. These characterizations clearly show that the polysaccharide in this fraction is amylose. In contrast, the *ae* alleles differed significantly in the physicochemical properties of the butanol noncomplexing fraction (Table III). In general, *i1* and *i2* differed from all other *ae* alleles, although occasionally these two alleles did not differ significantly from one other *ae* allele.

The β-amylolysis limit of the noncomplexing fraction was lower than that of the complexing fraction (Tables II and III), but higher than the 57.7% conversion of *wx* maize starch (100% amylopectin). Thus, this material is not entirely amylopectin; however, more branch points are present than found in the complexing fraction. The characterizations in Tables II and III are in agreement with comparable β-amylolysis limits, indicating that the noncomplexing fraction has at least been enriched for the anomalous component found in *ae* starch.

To further characterize the material, the ratio of total to reducing glucose units was determined (Table III). Chain length would be estimated by this procedure if no branched polysaccharide were present. The differences observed, considered with the β-amylolysis limits, indicate that *i1* and *i2* butanol noncomplexing polysaccharides have an increased number of branches compared to those of the other *ae* alleles. A slight increase in branching would be expected to dramatically increase the apparent chain length, since *wx* starch has no reducing groups detectable by this method. The fact that the ratios for the noncomplexing component are lower, and not higher than those of the complexing component, indicates that considerable short-chain linear material is present.

The amylose content of the noncomplexing fraction, as estimated by two different procedures, indicates considerable linear material is present (Table III). Linear glucose molecules with a $\overline{DP}$ less than 135 have an absorbance maximum below 615 nm (23,24) which is the wavelength used in these blue value procedures for amylose determination. Thus, assuming material with $\overline{DP}$ less than 135 is present, these amylose percentages may be too low. The slightly higher amylose percentages calculated from the I_2-KI absorbance spectrums *vs.* those based on the KI-KIO_3 method (Tables II and III) resulted from I^- being limiting in the latter procedure. This effect is minimal because parallel tests comparing the KI-KIO_3 method we used with the KI-KIO_3 procedure as detailed by Wolf *et al.* (15) resulted in only an average 2% increase for various types of starch samples with the KIO_3 amount reduced sixfold.

From the absorbance maximum, the $\overline{DP}$ can be estimated for linear amylose molecules by the procedure of Banks, Greenwood, and Khan (24). Using 590 nm in their equation, the $\overline{DP}$ was calculated to be 75. Similarly, a linear polyglucose molecule ($\overline{DP} = 61$) was experimentally shown to have an absorbance maximum of 590 nm (23). Since amylopectin has little absorbance at this wavelength, the noncomplexing fraction appears to contain similar linear material.

The 60% of the starch not solubilized by aqueous leaching was dispersed with three DMSO extractions, and the amylose content was estimated. The residual granules from *i1* and *i2*, 35.4 and 39.3% amylose respectively, had significantly less amylose than those from *B2, B3, M1, M2,* and *Ref* which averaged 48.7%. Considering all *ae* alleles, the amylose content of the residual granules was 14–20% less than that observed in the unleached starches. Thus, amylose was

preferentially extracted by aqueous leaching; however, as reported by others (7,12), not all linear material was leached.

From these characterizations (Table III), the butanol noncomplexing fraction appears composed primarily of linear polysaccharides; however, some branching is present. The nature and distribution of the branching cannot be determined from these results. The differences between *il* and *i2* and the other *ae* alleles can be explained if a small but constant amount of standard amylopectin is assumed to be leached. The *il* and *i2* alleles contain less amylose (Table I), and less linear butanol noncomplexing polysaccharide appears to be extracted by leaching. Thus, a constant amount of amylopectin would have a greater influence on measured physicochemical parameters for *il* and *i2* than for the other five *ae* alleles. Of course, if the noncomplexing fraction is composed of polysaccharides with occasional branch points as suggested by Mercier (9), slight variation in the extent of branching may be measured instead.

Since the apparent amylose content is lower in *il* and *i2* compared to the allele used in amylomaize production, the allele used in amylomaize hybrids cannot be replaced by these alleles to obtain increased amylose content for industrial purposes. In addition, none of these alleles appears to contain a new starch type with specialized properties differing from those currently available from amylomaize starch. Instead, these starches probably vary in levels of the same components, with starches of lower apparent amylose content containing less short-chain polysaccharide.

The observed differences in the starches examined in the present study may be due to effects of the alleles *per se*, to residual genetic differences remaining in the backcrossed material, or to chance environmental effects. Environmental influences are unlikely since the results were in good agreement for the four ears examined for each W64A *ae* allele. Residual genetic differences are unlikely, because theoretically more than 99% of the genetic material is that of a common inbred, and because similar results were observed in backcross conversions to both the W64A and W23 inbreds.

Other *ae* alleles not sufficiently backcrossed to be included in this investigation might have different properties; however, the similarity of *B2, B3, M1, M2,* and *Ref* suggests this is unlikely. Heteroallelic *ae* forms in the transconfiguration might be associated with unique starch properties, although the phenotypic similarity between heteroallelic and homoallelic kernels[3] indicates differences would be small. Stocks homozygous for any two of these *ae* alleles might have different properties; however, breeding such an *ae* double mutant is currently impossible.

[3]R. G. Creech, unpublished observations.

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